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Presence of integrase core domain in mutant type black pepper *Piper nigrum* L. 'Thekken' with altered inflorescence architecture

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Black pepper (*Piper nigrum* L.), referred as the 'King of Spices', is native to India and has shown declined productivity over the years. It exhibits diverse quantitative and qualitative traits, particularly in spike length, floral composition, floral arrangement, fruit size and number. The novel mutant variety of black pepper (*Piper nigrum* L.) 'Thekken' shows a remarkable branching character in the spikes. In the present study, we analyzed *RAMOSA3* (*RA3*) gene at the molecular level in this variety for yield improvement. Screening using degenerate primers designed for *RA3* was carried out in 'Thekken' and a non-branching variety 'Karimunda' at the genomic level and at different stages of spike development at the transcriptome level. Sequence analysis of the amplicons generated in RT-PCR revealed the presence of an integrase core domain in the mutant type of black pepper, suggesting a possibility of mutation at this locus in the branched variety due to retrotransposon integration. The study suggests a possibility for introgression of the genes responsible for branching trait from the mutant variety of black pepper type 'Thekken' to other conventional cultivated varieties of black pepper that show single unbranched spike thereby increasing the productivity of black pepper which has very high economic value and export potential.

Keywords: Mutation, Spike branching

Black pepper (*Piper nigrum* L.) is a highly priced commodity mainly used as spice (also referred to 'King of Spices') and in traditional medicines[1,2]. There is a drastic decline in the production and productivity of black pepper in India over the years. Indian pepper production is projected to decline in 2019 to 47,000 tonnes from 64,000 tonnes in 2018[3]. India is the centre of origin of black pepper and lot of species diversity is reported in quantitative and qualitative traits[4,5]. Diversity is exhibited in spike length, floral composition, floral arrangement, fruit number, size, etc[6].

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Normally, the spikes of black pepper are unbranched. However, a mutant type of black pepper 'Thekken' is reported to exhibit remarkable spike branching trait (Fig. 1). There are about 30 to 40 branches per spike which gives 300 to 400 berries per spike in contrast to 150 to 200 berries in normal unbranched spikes of other varieties[7,8]. The yield is 8 kg/plant in 'Thekken' compared to 4 kg/plant in 'Karimunda'.

Spike branching trait is of great economic significance as it can contribute to high yield. Modifications in inflorescence architecture is reported in different crop species, such as *Oryza sativa*, *Arabidopsis thaliana* and *Zea mays* due to mutations in different genes *viz*. *LEAFY*, *APETALA1*, *TERMINAL FLOWER 1*, *FLOWERING LOCUS T*, *RAMOSA* family genes, etc[9-13].

Inflorescence branching appears to be largely regulated through the *RAMOSA* gene network, and the name '*ramosa*', originates from the Latin word '*ramus*' means branch, and reflects the phenotype of the *ra* mutants, which have a highly branched inflorescence. The *RAMOSA* family mainly involves three genes, among them *RAMOSA3* (*RA3*) encoding trehalose 6 phosphate phosphatase is reported to cause altered inflorescence architecture in *Zea mays*[14].

To commercially exploit the spike branching trait, a better understanding of its molecular mechanism is essential at the genome and transcriptome level. The only information on inflorescence architecture related gene in black pepper is the report on a sequence

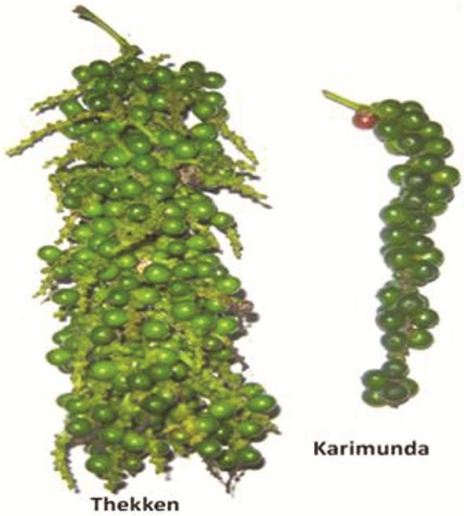

Fig. 1 — Spikes of type 'Thekken' and control variety 'Karimunda'

homologous to *TFL1* gene in 'Thekken'[15]. Hence, the present study was taken up to screen the presence of *RA3* at the genome and transcript level using PCR and RT-PCR to understand the molecular mechanism for induction of spike branching in 'Thekken' to aid in the yield improvement.

Materials and Methods

Sample collection

The samples *viz*., leaves and spikes were collected in liquid nitrogen from the plants ('Thekken' and control variety 'Karimunda') maintained in the farmer's field at Idukki district, Kerala, India and stored at –80°C.

DNA isolation

DNA was isolated from the leaves by standardized protocol reported by Subba *et al.*[16] and stored at –20°C for further studies.

RNA isolation

Total RNA was isolated from spikes at three developmental stages *viz*. Stage1 (1 cm), Stage 2 (4 cm) and stage 3 (9 cm), using Trizol reagent (Invitrogen, USA). All the materials used for RNA extraction were treated with 3% hydrogen peroxide overnight and autoclaved twice to reduce RNase contamination. The quality and quantity of DNA and RNA extracted were checked using UV spectrophotometer.

PCR

Forward and reverse degenerate primers were designed for the candidate gene *RA3* using Primer3 and oligocalc as per the details provided in Table 1. PCR amplification of the genomic DNA was carried out using the designed degenerate primers. PCR was performed in 25 µL PCR reaction mixture consisting of 1X PCR buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 70 pM each primer, 50 ng DNA, 1.5 U *Taq* DNA polymerase and nuclease free water.

Reverse Transcriptase-PCR

RT-PCR was performed in two steps *viz*., cDNA synthesis and PCR with designed primers. cDNA was synthesized by using First strand synthesis kit (Invitrogen, USA) and PCR was performed as described above. Presence of cDNA was confirmed by using ubiquitin gene (housekeeping gene) specific primers[15].

Gel elution and sequencing

Amplicon obtained from RT-PCR was eluted from agarose gel and sequenced at regional facility at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala

Sequence analysis

Homology search of the sequence was done using online bioinformatics tools tBLASTx and BLASTn. Percent identity of the sequence obtained to the reported sequence of *RA3* in NCBI database was carried by multiple sequence alignment using Clustal Omega and the presence of conserved domains in the sequence obtained was analysed using Conserved Domain Database search (CDD).

Results and Discussion

Studies on molecular basis of inflorescence architecture in black pepper is scarce[15]. Possibly, the present study is the first report of presence of an integrase core domain in black pepper. Good quality of DNA and RNA was obtained as revealed by distinct bands in agarose gel electrophoresis. The A_{260}/A_{280} for DNA samples ranged between 1.6 and 1.9 and for RNA it ranged between 1.6 and 2.3. Intact band of expected size of 99 bp was obtained from cDNA by RT-PCR using ubiquitin primers indicating good quality of the isolated cDNA in all the stages in both the varieties.

PCR using genomic DNA as template, produced two bands of size ~450 bp and ~650 bp with *RA3* gene primers in both 'Thekken' and control 'Karimunda' variety (Fig. 2). This may be due to the degeneracy of the primers used in the study.

RT-PCR amplified ~415 bp amplicon in Stage II of branching type of black pepper (Thekken) (Fig. 3). There was no amplification in any other stages of 'Thekken' as well as 'Karimunda'. The sequence of the eluted amplicon is given in Table 2 and has been

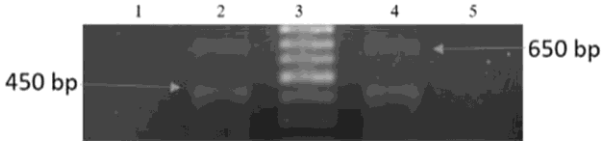

Fig. 2 — Agarose gel electrophoresis of PCR product from genomic DNA using *RA3* primers. [Lanes: 1, Template blank; 2, Thekken; 3, 100bp ladder ; 4, Karimunda; 5, Primer blank]

Table 1 — Primers designed for *RA3* gene

Gene		Sequences	Length	% GC	Tm
RAMOSA3	F	GSAAGCARATMGTGATGTT	19	42	51.8
	R	GACCTGACCTCCTCGTTCA	19	58	54.6

[Multiple sequence alignment- gi: 162459858, 787035541; Standard nucleotide coding system: S= C, G; R= A, G; M= A, C]

Table 2 — Sequence of amplicon obtained by using *RA3* primers in RT PCR of Stage II spikes of 'Thekken'

5' ATGAAATTTTGCTTTTCATCCTCTCCTTAACTTGCTTATGTAACTTCTTCACCAAATCTGCCTTAGAATTAGCATCAACAC
TCACAAAATTGTTAGGTGGCAATGGCAATAAATCAATAGGGGTGAGTGGATTAAAACCATAAACAATCTCAAATGGAGA
ACAATGAGTAGTGCTATGAATAGCCCTATTGTAAGCAAACTCCACAAATGGTAAACAATCCTCCCATGTCCTAATGTTCT
TTTCTATGATTGCACTAAGCAAAGTAATCAAAGTCCTATTAACTACTTCAGTTTGCCCATCTGTTTGAGGGTGACAAGTAG
TTGAAAATAATAGCTTAGTTCCTAACTTTCCCCACAACACACGCCAAAAATGACTCAAAAACTTAACATCACTATCTGCT
TCCCACAGG 3'

Fig. 3 — Agarose gel electrophoresis of PCR product from cDNA using *RA3* primers. [Lanes: 1, Stage I (Karimunda); 2, Stage II (Karimunda); 3, Stage III (Karimunda); 4, 100bp ladder; 5, Stage I (Thekken); 6, Stage II (Thekken); 7-Stage III (Thekken)]

deposited in the NCBI database (Accession number-KX518738). Varied amplification in the cDNA of Stage II from 'Thekken' and 'Karimunda' during RT-PCR using the designed primers suggests that altered expression of the region under study may play a role in the induction of spike branching in 'Thekken'. tBLASTx analysis showed 3574 hits and the best match was with chromosome 7 of *Cucumis melo*. With BLASTn, 52 hits were obtained showing similarity to uncharacterized mRNA sequence from *Brassica napus* (LOC106408221). Clustal Omega analysis showed 39.21 and 40.94 percent identity with the reported sequences of *RA3* of *Zea mays* and *Vitis vinifera* in the NCBI database, and these two reported sequences among themselves showed 61.30 percent identity. On CDD search, sequence of the amplicon expressed during Stage II in 'Thekken' exhibited the presence of an integrase core domain on CDD search [Specific hit, e-value = 3.06e-05, pfam00665]. Integrase mediates integration of a DNA copy of the viral genome into the host chromosome. Such domains are reported to be particularly abundant in plant genomes and are intimately involved in the evolution of genome structure and size[17,18].

Retrotransposons are mobile elements which replicate by intermediary RNA using reverse transcriptase enzyme[19,20]. They are widely dispersed in plant and animal genomes and contribute to genome evolution by causing a broad spectrum of mutations, reorganizing genomes, and contributing to the physical size of the host genome[21].

In wheat, transcriptional activation of retrotransposons alters the expression of adjacent genes, and read-through transcription from retrotransposons is associated with the activation or silencing of flanking genes[22].

The study suggests that retroviral integration can be a possible reason for the altered inflorescence architecture in the novel mutant variety of black pepper 'Thekken'. Co-expression studies for synergistically expressed genes/transcripts and its correlation with agronomic traits for yield can further validate the results of the study.

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Conflict of interest

The authors declare that they have no conflict of interest.

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